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14. ABSTRACT

Over the funding period substantial progress was made towards completion of the project goals described in the original statement of work. AIF deficient PC3 cells were established and used to demonstrate that AIF does not affect the in vitro growth of these cells but is critical for growth of these cells in a xenograft model. These growth discrepancies manifest in both three dimensional cell culture growth and invasion assays, in which AIF was shown to be necessary for normal growth and invasion. Interestingly, three dimensional growth required the enzymatic activity of AIF, as an AIF variant lacking enzymatic activity failed to rescue cell growth in these cultures. Extending beyond our statement of work we have also shown that AIF ablation results directly in a loss of complex I of the mitochondrial electron transport chain with commensurate increases in cellular glycolytic activity. Noteably, while the enzymatic activity of AIF was not necessary to restore complex I protein levels, glycolytic levels remain elevated in all but the wildtype instance. Overall these data demonstrate for the first time a direct role for AIF in the progression of prostate cancer, and suggest that one mechanism that may explain this role is through control of mitochondrial energy production as a direct result of AIF enzymatic activity.

15. SUBJECT TERMS
Prostate Cancer; X-linked Inhibitor of Apoptosis; Apoptosis Inducing Factor

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Introduction

As the second most common form of cancer diagnosed among men in the western world prostate cancer represents a significant healthcare threat, and there is great need for new and more effective treatments for this disease. This research project seeks to determine the potential role of two proteins, apoptosis inducing factor (AIF) and X-linked inhibitor of apoptosis (XIAP), in prostate cancer pathogenesis. Both proteins are multifunctional, and one of our goals is to determine which properties of each molecule are important for disease progression. This report summarizes progress towards completing these research goals during the three year funding period.

Body

We have made significant progress towards completing the tasks outlined in our original Statement of Work, as summarized below:

Task 1a. To determine the effects of AIF and XIAP on tumorigenesis.

- **Generation of PC3-derived cell lines that stably lack expression of endogenous AIF, XIAP, or both (Months 1-9).** PC3 cells were successfully infected with lentiviruses containing RNAi sequences targeting AIF and XIAP. Stable, clonal cell lines were isolated using fluorescence activated cell sorting, and suppression of protein expression has been determined by immunoblot analysis (Supporting Data Figure 1). Nearly complete ablation of XIAP and AIF protein expression has been achieved. Control and AIF-deficient cells have been evaluated both for in vitro growth (Figure 2) and for differences in sensitivity to a panel of chemotherapy drugs (Figure 3). No differences in the in vitro growth rates or sensitivity to chemotherapeutics were observed between AIF-deficient and AIF-proficient cells.

- **Assessment of tumor progression (Months 10-18).** Control and AIF-deficient PC3 cells were implanted subcutaneously into athymic nude mice. Tumor progression of these cells was followed over time, and we observed a substantial decrease in the rate of tumor growth of AIF-deficient cells compared to controls (Figure 4). Immunoblot analysis of tumor lysates confirmed that AIF protein expression was not reactivated during tumor growth (Figure 4).

Task 1b. To determine the specific properties of AIF and XIAP required for tumorigenesis.

- **Generation of PC3-derived "restoration" cell lines expressing AIF and XIAP variants lacking defined functions (Months 10-18).** We initially succeeded in producing lentiviruses based on the FG9 backbone vector containing sequences to express wildtype AIF and the AIF variants T263A/V300A (TVA mutant) and K255/265/510/518A (K4A mutant). As described in our original proposal, the TVA mutant lacks enzymatic activity but retains death inducing functions, whereas the K4A mutants retains enzymatic activity but fails to induce cell death (Urbano et al., 2005). These viruses were used to subject AIF-deficient PC3 cells derived above to a second round of lentivirus infection. For reasons that remain unclear, we were unsuccessful at using these lentiviruses to stably infect AIF deficient cells in order to generate "restoration" cells lines with these AIF variants. While preliminary results suggest viral infection was successful, we were unable to achieve stable incorporation of AIF cDNA contained within these viruses. Our hypothesis was that the lentiviral backbone is too large for efficient viral packaging. With this hypothesis in mind, we changed the lentiviral backbone used for the production of lentivirus to a smaller construct than described in our previous efforts, and this new

approach was proven successful for the establishment of PC3-derived cells that express the wildtype and TVA variant described above. Immunoblot analysis indicated restored expression is shown in Figure 5. Interestingly, we have been unable to achieve expression of the K4A mutant, likely due to toxicity associated with stable expression of this protein. As our hypothesis remains that the enzymatic activity of AIF is more important than death induction, we proceeded without the K4A variant in our subsequent experiments.

- **Assessment of the PC3-derived tumor progression (Months 18-27).** Due to delays in establishment of these cells as described above, these experiments were substantially delayed. While completion of this task will not be achieved during the funding period, experiments are in progress and we are hopeful of obtaining useful data from their completion. As a preface to and/or surrogate for these studies, we examined the ability of these cell lines to grow in three-dimensional cell culture experiments, which indicate that only the wildtype AIF protein, and not the TVA variant, is capable of supporting normal three dimensional growth of shAIF-PC3 cells (Figure 8).

Task 2. To investigate the contribution of AIF to tumorigenesis in a transgenic murine model of cancer**.**

- **Establish a breeding colony of prostate-specific *Pten*-deficient mice (Pten$^{-/-}$) (Months 18-24).** A breeding colony was established at the beginning of year three of study.

- **Breed Pten$^{-/-}$ with AIF-deficient (Hq) mice (Months 24-30).** Due to delays in other aspects of this study (see above) these experiments have yet to begin. They are on schedule to be initiated in the year future but after completion of the three year funding period of this study.

- **Evaluation of tumor progression/burden in Pten$^{-/-}$/Hq mice (Months 30-36).** Similar to the Hq Pten$^{-/-}$ breeding experiments above, these experiments have yet to begin. They are on schedule to be initiated in the year future but after completion of the three year funding period of this study.

In addition to the progress described above relating to the original statement of work, we have made additional progress related to understanding the role of XIAP and AIF in the control of prostate cancer pathogenesis. Data presented shown in Figures 2 and 4 suggested that whereas AIF deficient cells displayed no differences in cell growth characteristics when cultured in vitro, these cells are significantly attenuated in their ability to grown in vivo. These data led us to investigate the ability of AIF to support the ability of prostate cancer cells to grow in 3D culture and invade basement membrane. When plated in culture dishes coated with Matrigel basement membrane, parental and control PC3 cells grow well and display macroscopic structures (Figure 6). AIF-deficient cells not only fail to display macroscopic structures seen with parental and control cells, they appear round up and detach from the dish, indicative of cell death (Figure 6). When the invasive properties of these cells were investigated, we observed that control cells displayed substantial invasive growth (Figure 7). However, AIF-deficient cells were completely repressed in their ability to invade through Matrigel (Figure 7). These data suggest that the mechanism by which AIF supports prostate cancer tumorigenesis is through allowing cells to survive and invade the extracellular milieu.

Stemming from our observations shown in Figure 8, that the enzymatic activity of AIF is necessary for tumorigenic growth, we have assessed the affects of AIF ablation upon expression of the mitochondrial electron transport chain component, complex I (Figure 9). Ablation of AIF alone in PC3 cells leads to a substantial reduction in complex I protein levels when compared to controls. Interestingly, and in contrast to our three-dimensional growth experiments, the TVA variant is able to restore normal complex I expression to shAIF cells. However, when glucose consumption was assessed, we noted that whereas AIF ablated cells display substantially higher levels of glucose consumption, only wildtype AIF and not the TVA variant was able to restore glucose consumption back to control levels (Figure 10).

An open question related to the overall activity of AIF in prostate cancer cells was whether the death inducing capabilities of AIF had a role to play in cancer progression. To answer this question, we assessed the ability of the DNA alkylating agent MNNG, reported by previous studies to induce an AIF-dependent cell death pathway in a variety of other cell types, to induce cell death in PC3 cells either lacking AIF, or overexpressing either wildtype or TVA AIF. As shown in figure 11, neither the ablation nor overexpression of AIF in PC3 cells resulted in a change in sensitivity to MNNG treatment.

Key Research Accomplishments

- PC3 cells deficient in AIF, XIAP, and both proteins were established
- It was determined that the loss of AIF does not affect the in vitro growth characteristics of PC3 cells
- AIF protein ablation had no effect on the sensitivity of PC3 cells to a wide range of cell death-inducing stimuli
- It was shown that loss of AIF dramatically slows the growth of PC3 cells when examined in a mouse xenograft tumor growth model
- Lentiviruses containing expression sequences for the AIF mutants T263A/V300A and K255/265/510/518A were created
- AIF-deficient PC3 cells have been infected with lentiviruses containing the AIF variants T263A/V300A and K255/265/510/518A
- Restoration/overexpression of both wildtype and AIF-TVA has been achieved in our panel of PC3 cell lines
- It was demonstrated that PC3 cells deficient in AIF are attenuated in their ability to growth in three dimensional Matrigel cultures
- PC3 cells deficient in AIF were shown to be substantially reduced in their ability to grow invasively in culture.
- Matrigel growth of PC3 cells as a consequence of AIF protein expression was assessed
- The necessity of AIF for maintenance of complex I protein levels in PC3 cells was demonstrated
- The impact of AIF protein expression upon glucose consumption was assessed
- The lack of AIF dependence to MNNG-induced cell death was definitively shown

Reportable Outcomes

None to date

Conclusions

Based on the progress so far, current conclusions include: 1) loss of AIF in PC3 cells has no affect upon the in vitro growth characteristics of these cells, 2) AIF does not contribute, either positively or negatively, to the death of PC3 cells induced by a variety of cell death stimuli, 3) loss of AIF severely compromises the ability of PC3 cells to form and grow tumors in a mouse xenograft model 4) AIF directly contributes to the ability of PC3 cells to grow and survive in the

context of three dimensional cultures, similar to results observed in our in vivo tumor growth studies, 5) AIF additionally supports the invasive growth of PC3 cells, 6) The enzymatic activity of AIF is necessary for PC3 cells to growth in three dimensional culture, 7) complex I expression requires AIF but is not dependent upon AIF enzymatic activity, 8) glucose consumption is elevated following AIF ablation, only wildtype AIF is capable of restoring glucose consumption back to normal levels, and 9) AIF plays no role in controlling the death of PC3 cells following treatment with the DNA alkylating agent MNNG.

Overall these conclusions suggest that in androgen-independent prostate cancer cells (PC3) AIF is not required for growth in vitro but is critical for normal tumor growth in vivo, and are consistent with the hypothesis that AIF is a relevant target for molecular therapeutic intervention. Moreover, AIF is critical for establishing tumorigeneic potential, and that this is achieved through support of invasion. These properties of AIF as a tumorigenic contributor are dependent upon the NADH-oxidase activity of the protein, though levels of complex I in mitochondrial do not depend directly upon this activity, only upon the presence of the AIF protein.

References

Urbano, A., Lakshmanan, U., Choo, P.H., Kwan, J.C., Ng, P.Y., Guo, K., Dhakshinamoorthy, S. and Porter, A. (2005) AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. *Embo J*, **24**, 2815-2826.

Supporting Data

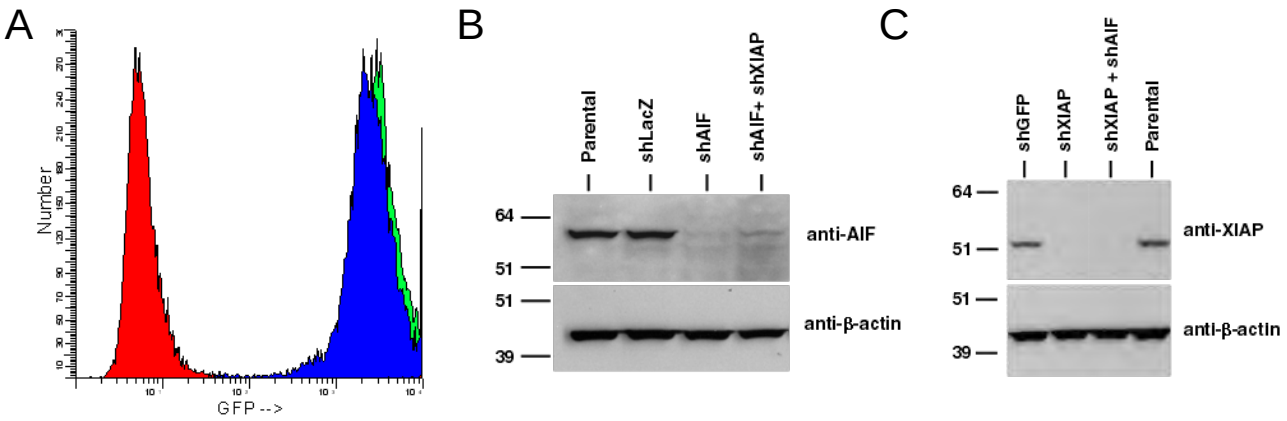


Figure 1. Establishment of AIF-Deficient PC3 Cells. PC3 cells were infected with control (shLacZ, shGFP), shAIF containing, shXIAP containing, or both shAIF and shXIAP containing lentiviruses. GFP (shLacZ, shAIF) and dsRed (shGFP, shXIAP) positive cells were then sorted by flow cytometry. A) Representative flow cytometry histograms for parental (red), shLacZ (blue) and shAIF (green) PC3 cells. B) Anti-AIF immunoblot analysis of sorted cells demonstrating near complete ablation of AIF protein in cells infected by shAIF lentivirus. C) Anti-XIAP immunoblot analysis of sorted cells demonstrating near complete ablation of XIAP in cells infected by shXIAP lentivirus.

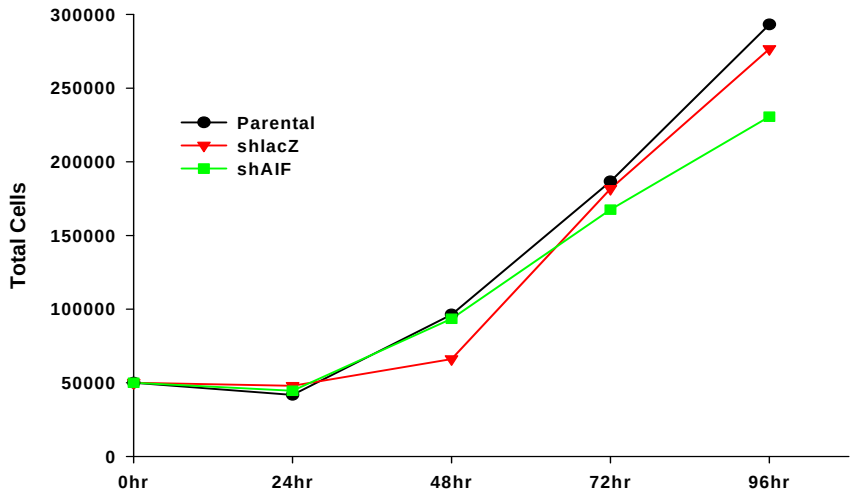


Figure 2. Loss of AIF does not affect growth rate of PC3 cells in vitro. Parental, shLacZ, and shAIF PC3 cells were seeded in replicate wells at identical densities in six well plates. Beginning at 24 hours and continuing daily, cells were harvested and total cell number was determined by Coulter counting. Data shown is representative of four independent experiments. As indicated by the data, AIF deficient PC3 cells grow at the same rate in vitro as parental and shLacZ control cells, suggesting that AIF protein is not required for normal growth in culture.

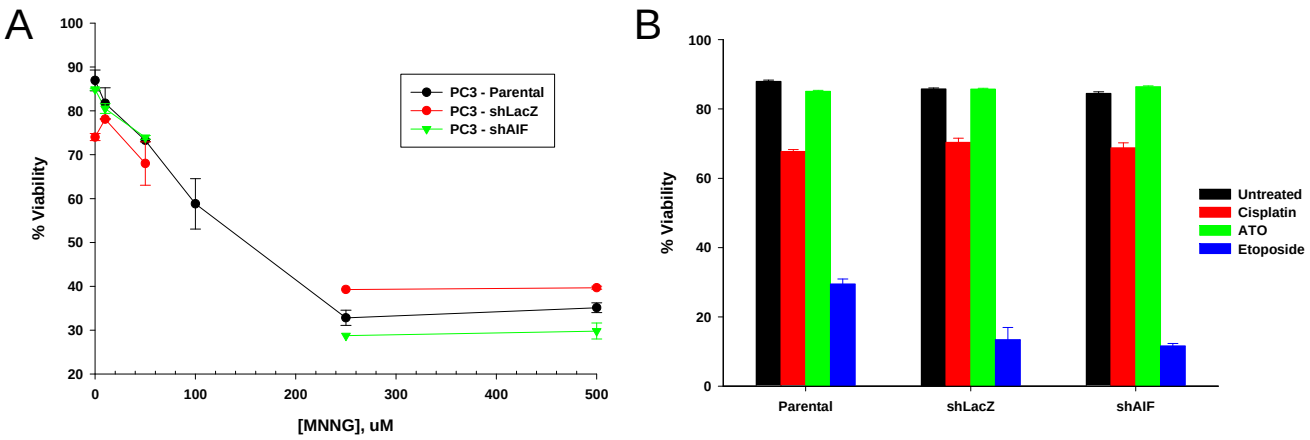


Figure 3. Sensitivity of PC3 cells to death-inducing stimuli is not altered by AIF ablation. A) Parental, shLacZ, and shAIF PC3 cells were treated with increasing concentrations of the DNA alkylating agent MNNG for 15 minutes. Cell were then washed and incubated overnight in normal growth media. Cell viability was then assessed by propidium iodide staining followed by flow cytometry. B) PC3 derived cells were treated overnight with the chemotherapy drugs cisplatin, arsenic trioxide (ATO) and etoposide. Viability was then determined as described in panel A. Note that AIF ablations has no effect on the sensitivity of PC3 cells to any of the treatments employed. These data suggest that AIF is not a critical component either for survival following drug treatment, or for the ability of these drugs to induce cell death.

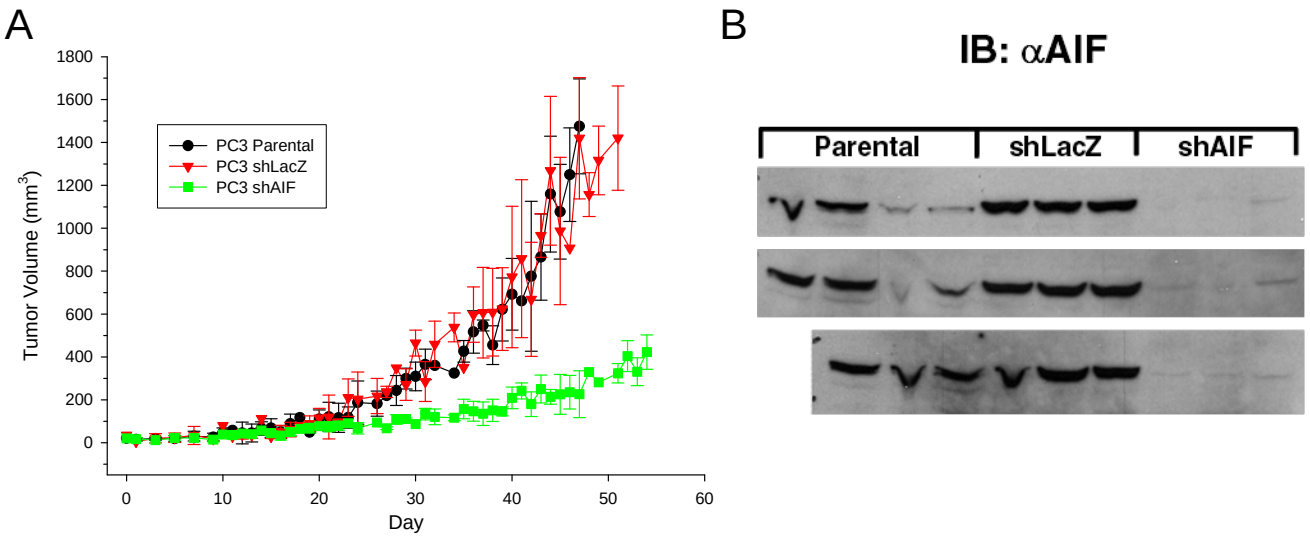


Figure 4. Growth of PC3-derived cells following implantation in nude mice. A) 3 x 10^6 parental (Black), shLacZ (red), or shAIF (green) PC3 cells were injected subcutaneously into the right hind flank of athymic nude mice. Subsequent tumor growth was followed by caliper measurements three times weekly until animals reached criteria for removal from study. Note the substantial decrease in tumor growth observed in AIF-deficient PC3 cells. B) Tumors were harvested, protein extracts were prepared, and AIF protein expression in developed tumors was determined by immunoblot analysis.

Supporting Data

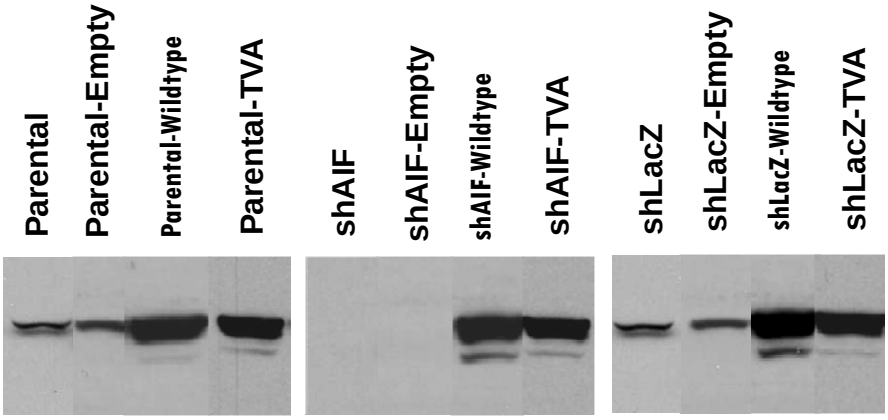


Figure 5. Restoration of AIF in shAIF-PC3 cells, overexpression in control cell lines. Parental (left), shLacZ (right) and shAIF (center) PC3 cells were stably infected with either a control lentivirus (empty) or lentiviruses containing cDNA encoding either wildtype AIF or the enzymatically deficient AIF variant TVA. Stable integration was selected by growth in puromycin. After selection, cells were subjected to immunoblot analysis to determine AIF expression levels. Note overexpression of wildtype and TVA-AIF in parental and shLacZ cells, and restoration of expression in shAIF cells. Equal loading of protein lysates was determined by immunoblot analysis (data not shown).

Supporting Data

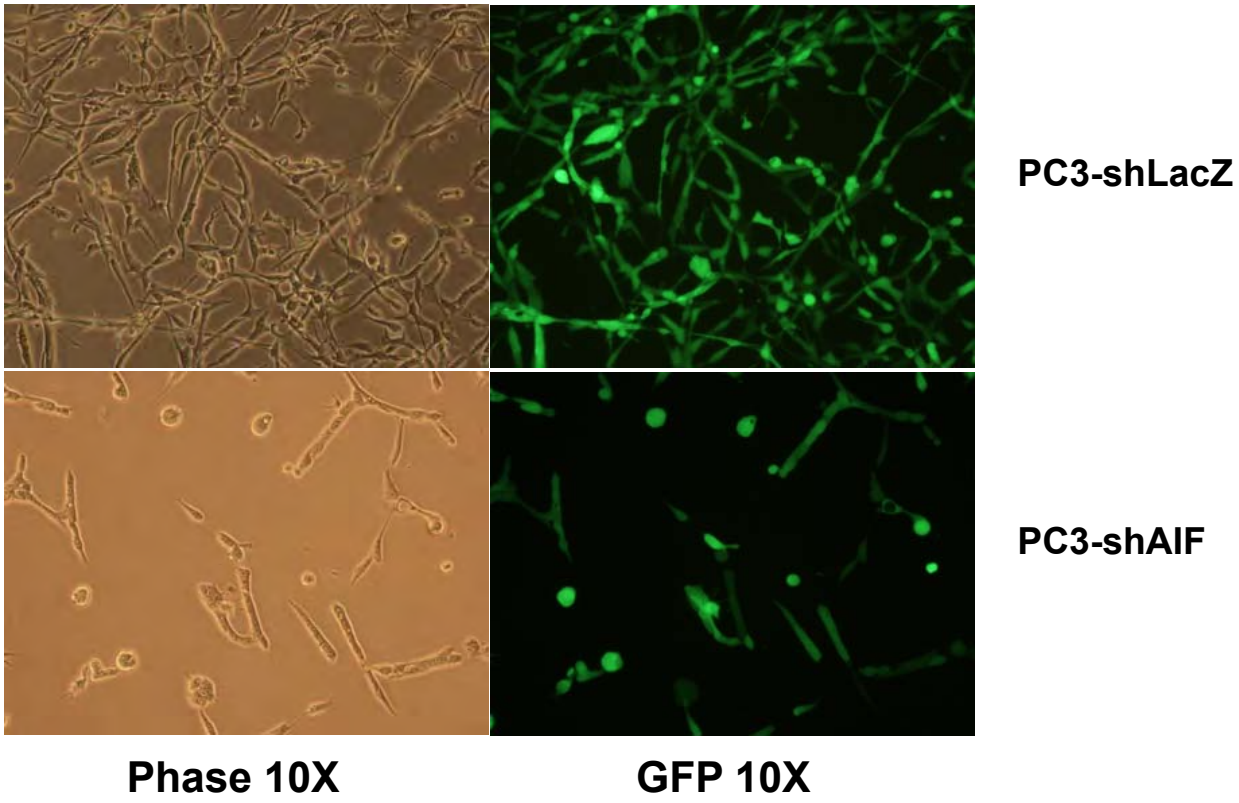
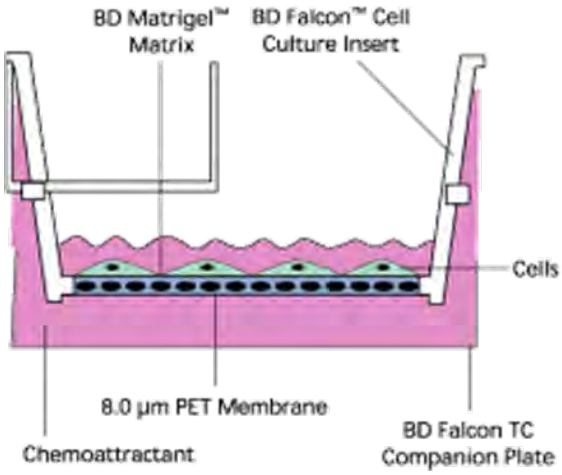


Figure 6. AIF-Deficient PC3 cells grow poorly on Matrigel basement membrane. Control (shLacZ, top row) and AIF deficient (shAIF, bottom row) PC3 cells were plated at 10,000 cells per well in 12-well culture dishes coated with a layer of Matrigel basement membrane. Cells were allowed to grow for 96 h, and then morphological features of cell growth were assessed by phase contrast microscopy (left column, magnification 10X). Since both cell lines express green fluorescent protein as a selectable marker for lentiviral infection, fluorescence microscopy was also employed (right column, magnification 10X. Note that only control cells were capable of effective growth and formation of macroscopic structures whereas AIF deficient cells were fewer in number and appeared apoptotic, based on spherical morphological characteristics.

A



B

	16h	40h	65h
shLacZ	1.65	40.69	43.53
shAIF	0.54	3.88	3.61

Figure 7. Invasive growth of PC3 cells is compromised following AIF protein ablation. A) Schematic representation of the invasion assay. RPMI-1640 growth media with full serum (10%) as chemoattractant was placed in the bottom of 24-well tissue culture plates. 8.0 micron PET membrane inserts either uncoated (as control) or coated with Matrigel Matrix were placed into each well. shLacZ or shAIF cells were harvested, suspended in serum free media, and added to the top of each insert. Following growth for the indicated amounts of time inserts were collected, fixed with paraformaldehyde, and allowed to dry. B) The number of cells present on each insert was then assessed by fluorescence microscopy. Percent invasion for each cell line was determined by the ratio: # cells per Matrigel insert / # cells per control insert. Note that whereas a substantial proportion of control cells displayed invasion after 40h (panel B, top row), virtually no invasion was observed for AIF-deficient cells (panel B, bottom row). These data suggest that AIF is critical for the ability of PC3 cells to grow invasively.

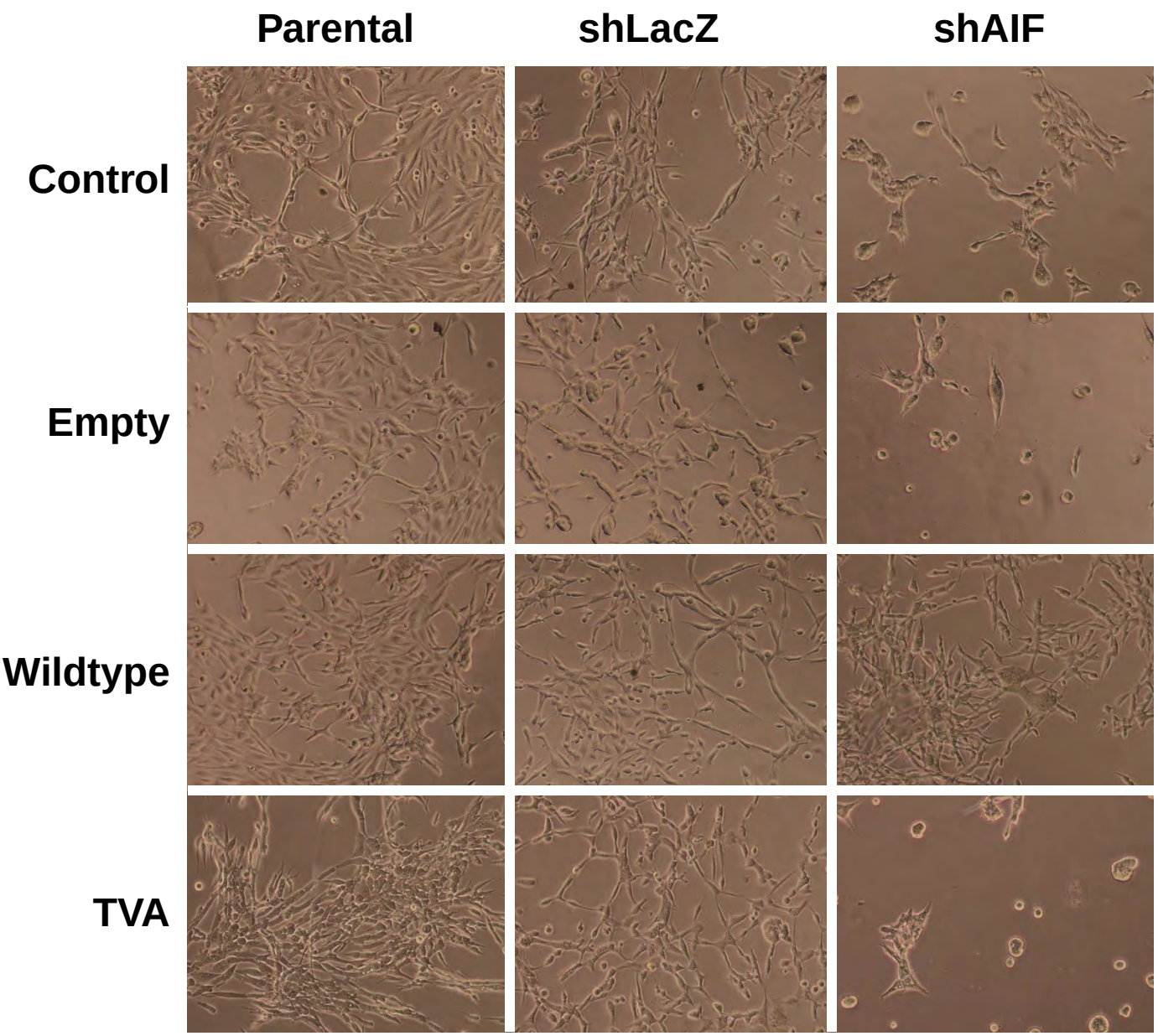


Figure 8. Matrigel growth of AIF-Deficient PC3 is restored by wildtype but not enzymatically deficient AIF. Parental (left column), shLacZ (center column) and shAIF PC3 cells (right column) restored infected with lentiviruses encoding the indicated AIF variants were plated at 10,000 cells per well in 12-well culture dishes coated with a layer of Matrigel basement membrane. Cells were allowed to grow for 96 h, and then morphological features of cell growth were assessed by phase contrast microscopy (magnification 10X). Note that shAIF cells (control, empty) were unable to grow in matrigel compared to parental and shLacZ cells, and that only restoration of shAIF cells with wildtype AIF was capable of allowing shAIF cells to grow. Further note that overexpression of either wildtype or TVA-AIF in parental and shLacZ cells had no observable impact on matrigel growth.

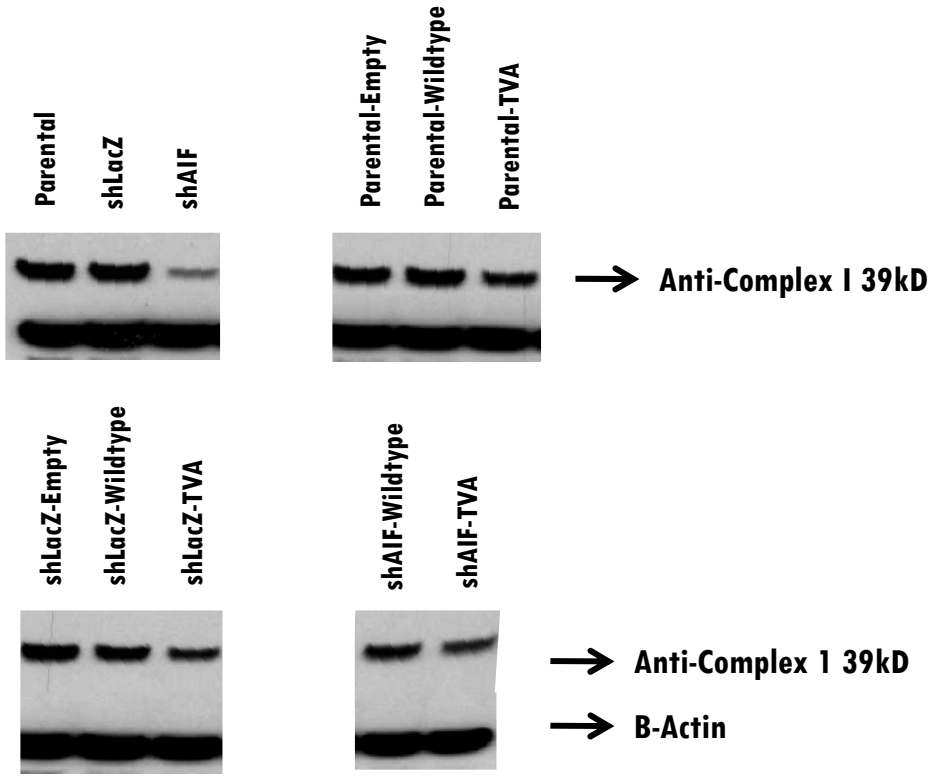


Figure 9. The 39 kDa subunit of Complex I is sensitive to AIF expression but not eznymatic activity. Whole cell lysates from derivatives of parental (top row) and both shLacZ and shAIF PCS cells (bottom row) were subjected to immunoblot analysis for the presence of the 39 kDa subunit of mitochondrial Complex I of the energy transport chain. Whereas AIF ablation in control shAIF cells leads to a substantial decrease in complex I (top row, left panel), restoration of these cells with either wildtype or TVA-AIF is sufficient to restore expression to normal levels (bottom row, right panel). Interestingly, whereas overexpression of wildtype AIF has no impact on complex I levels in parental or shLacZ cells, the expression of the TVA variant in these cells led to a slight suppression in complex I levels, suggesting that TVA may function as a dominant negative protein in these lines.

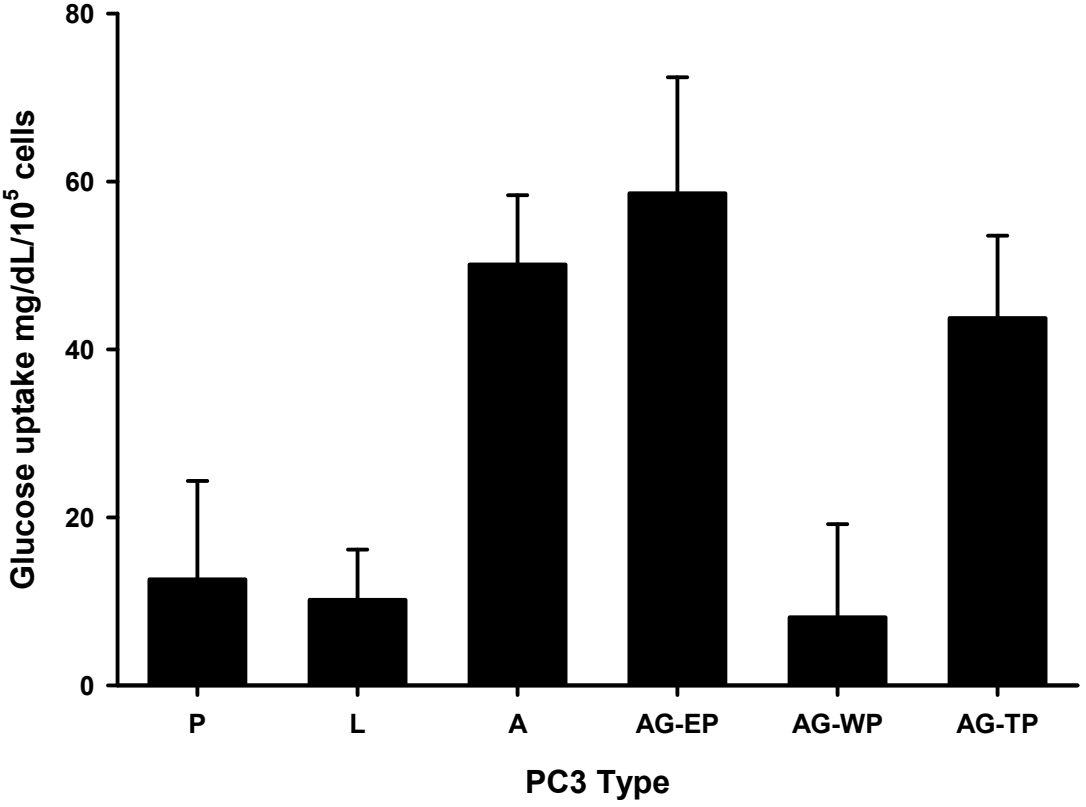


Figure 10. Glycolytic switch in AIF-Deficient PC3 cells is reverted only by restoration of enzymatically active AIF. Parental (P), shLacZ (L), shAIF (A), shAIF+empty (AG-EP), shAIF+WT (AG-WP), and shAIF+TVA (AG-TP) cells were seeded in 6-well plates and allowed to grow for 48h. Growth media was collected and the amount of glucose remaining was determined. Total cell number was determined by Coulter counting, and glucose consumption per cell was calculated. Note that AIF suppression in shAIF cells leads to a substantial increase in glucose consumption, consistent with an energetic switch to glycolysis for energy production, rather than oxidative phosphorylation. Also note that only wildtype AIF restored glucose consumption to normal levels; the shAIF cells expressing the TVA variant remained highly glycolytic.

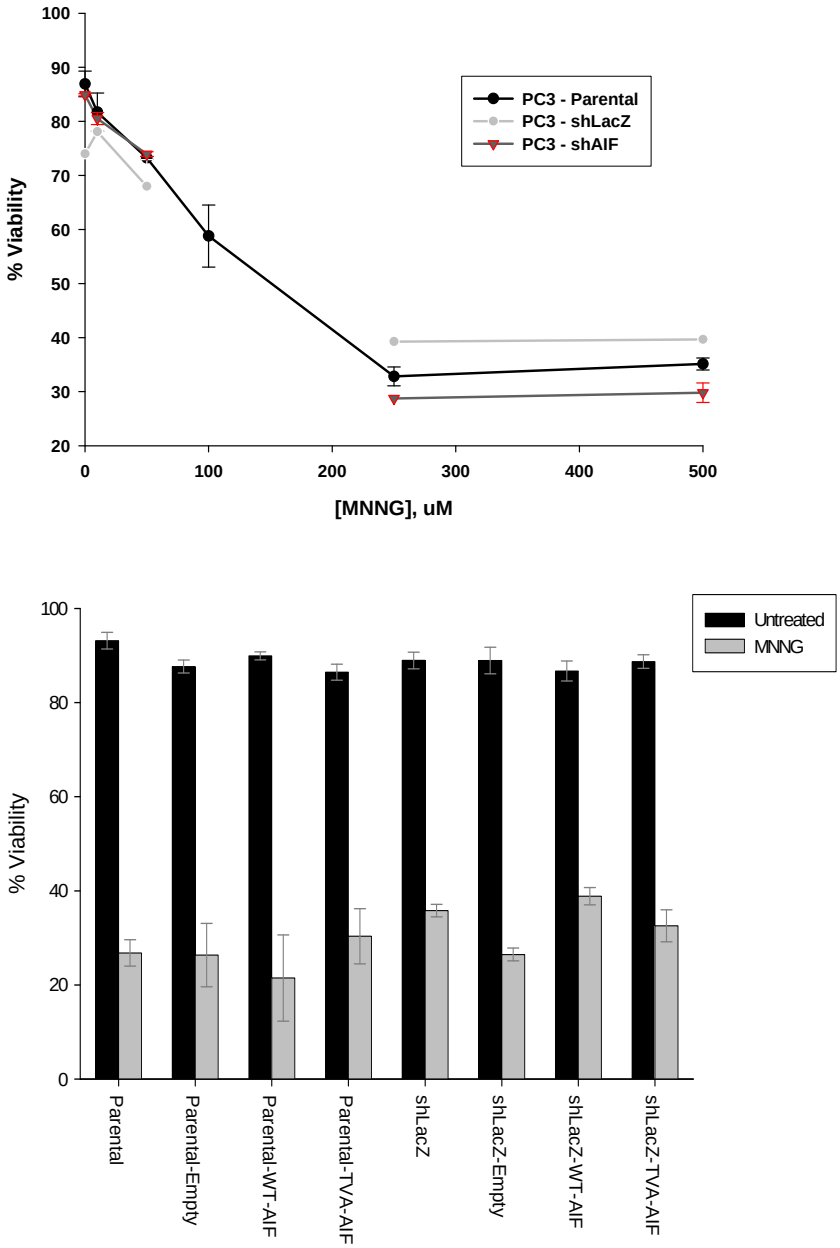


Figure 11. AIF is not involved in cell death mediated by the DNA alkylating agent MNNG. Top: Parental, shLacZ, and shAIF cells were treated with increasing concentration of MNNG for 15 minutes, drug was removed, and cells were incubated for an additional 20 h prior to determination of viability by propidium iodide staining and flow cytometry. Note that AIF ablation had no effect on the sensitivity of cells to MNNG treatment. Bottom: Parental and shLacZ cells overexpressing wildtype or TVA AIF were left untreated (black bars) or treated with MNNG (500 uM, gray bars) and analyzed as described above. Note that overexpression of neither wildtype nor TVA AIF affected the death or survival of parental and shLacZ cells.